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Update on Photosynthesis

How Do Algae Concentrate CO_2 to Increase the Efficiency of Photosynthetic Carbon Fixation?[1]

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The ability of photosynthetic organisms to use CO_2 for photosynthesis depends in part on the properties of Rubisco. Rubisco has a surprisingly poor affinity for CO_2, probably because it evolved in an atmosphere that had very high CO_2 levels compared with the present atmosphere. In C_3 plants the $K_m(CO_2)$ of Rubisco ranges between 15 and 25 μM. In cyanobacteria Rubisco has an even lower affinity for CO_2, and the $K_m(CO_2)$ can be greater than 200 μM. In comparison, the concentration of CO_2 in water in equilibrium with air is approximately 10 μM. From these numbers it becomes apparent that Rubisco is operating at no more than 30% of its capacity under standard atmospheric conditions. This is one of the reasons that C_3 plants contain such large amounts of Rubisco. Exacerbating this situation is the fact that O_2 is a competitive substrate with respect to CO_2.

In the atmosphere, where the O_2 level is 21% and the CO_2 level is 0.035%, the competition by O_2 accounts for as much as 30% of the reactions catalyzed by Rubisco. A number of photosynthetic organisms have developed ways to increase the level of CO_2 at the location of Rubisco in the plant. This results in an increase in CO_2 fixation and a decrease in the deleterious oxygenation reaction. An excellent example of a CO_2-concentrating mechanism in higher plants is C_4 photosynthesis, which has arisen independently in a number of plant families. Aquatic photosynthetic organisms such as the microalgae have also adapted to low CO_2 levels by concentrating CO_2 internally. This *Update* will focus on CO_2-concentrating mechanisms in the microalgae. For more detailed reviews of the CO_2 concentration by algae, the reader is referred to the special issue of the *Canadian Journal of Botany* (1998, Vol. 76) and the article by Raven (1997).

TYPES OF CO_2-CONCENTRATING MECHANISMS AND THE PROBLEM OF LEAKAGE OF ACCUMULATED CO_2

C_4 plants are the best-studied organisms that concentrate CO_2 to enhance the carboxylation reaction of Rubisco. They have high levels of PEP carboxylase in leaf mesophyll cells, whereas Rubisco is located primarily in the bundle-sheath cells. CA within the mesophyll converts CO_2 entering the leaf into HCO_3^-, which is the substrate for PEP carboxylase. The advantages that PEP carboxylase has over Rubisco are its high affinity for HCO_3^- and its insensitivity to O_2. At physiological CO_2 levels and pH, the HCO_3^- concentration in the cytoplasm of mesophyll cells is about 50 μM, whereas the $K_m(HCO_3^-)$ of PEP carboxylase is estimated to be about 8 μM. Therefore, in contrast to Rubisco, PEP carboxylase is saturated for HCO_3^- at ambient CO_2 levels. To finish the CO_2-concentrating effect of C_4 metabolism, the C_4 acid generated in the mesophyll cells is then transported to the bundle-sheath cells and decarboxylated, creating an elevated CO_2 level specifically within these cells.

The problem faced by all photosynthetic organisms that concentrate CO_2 is that it can easily diffuse through biological membranes. How can such a slippery substance be accumulated? In C_4 plants CO_2 is concentrated in specific bundle-sheath cells within the leaf. These are the only cells containing significant amounts of Rubisco. Here the thickened cell walls of the bundle sheath prevent the diffusion of the CO_2 generated by decarboxylation reactions. Microalgae face an additional problem in that they are composed of only one or a few cells, all with ready access to the environment; therefore, they must prevent the diffusion of CO_2 out of the cell while allowing the entry of other nutrients.

Microalgae overcome the problem of CO_2 diffusion by accumulating HCO_3^-. Being a charged species, HCO_3^- diffuses through membranes much more slowly than CO_2. However, because CO_2 is the substrate required by Rubisco, the accumulated HCO_3^- must be converted to CO_2 before C_i fixation takes place. This appears to be accomplished by packaging Rubisco within the algal cell and generating the CO_2 at that location through the action of a CA. A locally elevated CO_2 environment is thereby created in which CO_2 can out-compete O_2 at the active site of Rubisco. This allows the CO_2 to be used for photosynthesis before it can diffuse out of the cell. Thus, microalgae that concentrate CO_2 package Rubisco in a very specific location, have a means of concentrating HCO_3^-, and have a means of converting the accumulated HCO_3^- to CO_2 rapidly at the location of Rubisco.

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Abbreviations: ABC, ATP-binding cassette; CA, carbonic anhydrase; C_i, inorganic carbon.

THE LOCATION OF RUBISCO IN MICROALGAE

In higher plants Rubisco appears to act largely as a soluble protein that is distributed throughout the chloroplast stroma. By analogy, one might expect eukaryotic algae to have Rubisco throughout their chloroplast stroma and cyanobacteria to contain Rubisco throughout their cytoplasm, but this is clearly not the case. In most microalgae Rubisco is concentrated in a specific location: in carboxysomes in cyanobacteria and in the pyrenoid in algae (Fig. 1; Table I). Recent studies support the hypothesis that Rubisco localization is required for efficient acquisition of environmental CO_2.

Carboxysomes are electron-dense particles that are surrounded by a protein shell. Evidence that they contain large amounts of Rubisco is extensive. In fact, isolated carboxysomes have been found to be composed mostly of Rubisco (Price et al., 1992). Immunolocalization studies using antibodies raised against Rubisco indicate that the carboxysome is the primary location in cyanobacteria (McKay et al., 1993). A mutation that causes a 30-amino acid extension of the Rubisco small subunit leads to a Rubisco that does not pack into the carboxysome, which leaves the carboxysome empty (Schwarz et al., 1995). Mutations in any of the genes affecting the assembly, functioning, or shape of the carboxysome result in cells that cannot grow on air levels of CO_2 (Price et al., 1998).

Rubisco is also packaged in microalgae, where it is the major protein component of the pyrenoid. Pyrenoids have been purified from both *Eremosphera* (Okada, 1992) and *Chlamydomonas reinhardtii* (Kuchitsu et al., 1991), and in both cases they consisted primarily of Rubisco. In addition, *C. reinhardtii* cells with a mutation of the *rbcL* gene (Rubisco large subunit) that leads to a truncation of the large subunit of Rubisco have no pyrenoids (Rawat et al., 1996). Although it is accepted that Rubisco is the major constituent of the pyrenoid, there are conflicting findings regarding what percentage of the cell's Rubisco is in the pyrenoid. A recent report by Borkhsenious et al. (1998) demonstrated that in *C. reinhardtii* the amount of Rubisco in the stroma varies with growth conditions.

In all published immunolocalization studies the pyrenoid is densely labeled when an anti-Rubisco antibody is used as the primary probe (Borkhsenious et al., 1998). An example of this immunogold labeling is shown in Figure 1D. In these studies the amount of Rubisco in each subcellular location was estimated by multiplying the density of particles (particles per area) in that location by the average volume of the pyrenoid (2.4 mm^3) or the stroma (35.6 mm^3) (Lacoste-Royal and Gibbs, 1987). However, this still leaves a fairly broad range of estimates for the amount of Rubisco in the pyrenoid, from 50% to 99%. These differences could be attributed to the growth regime used by the various

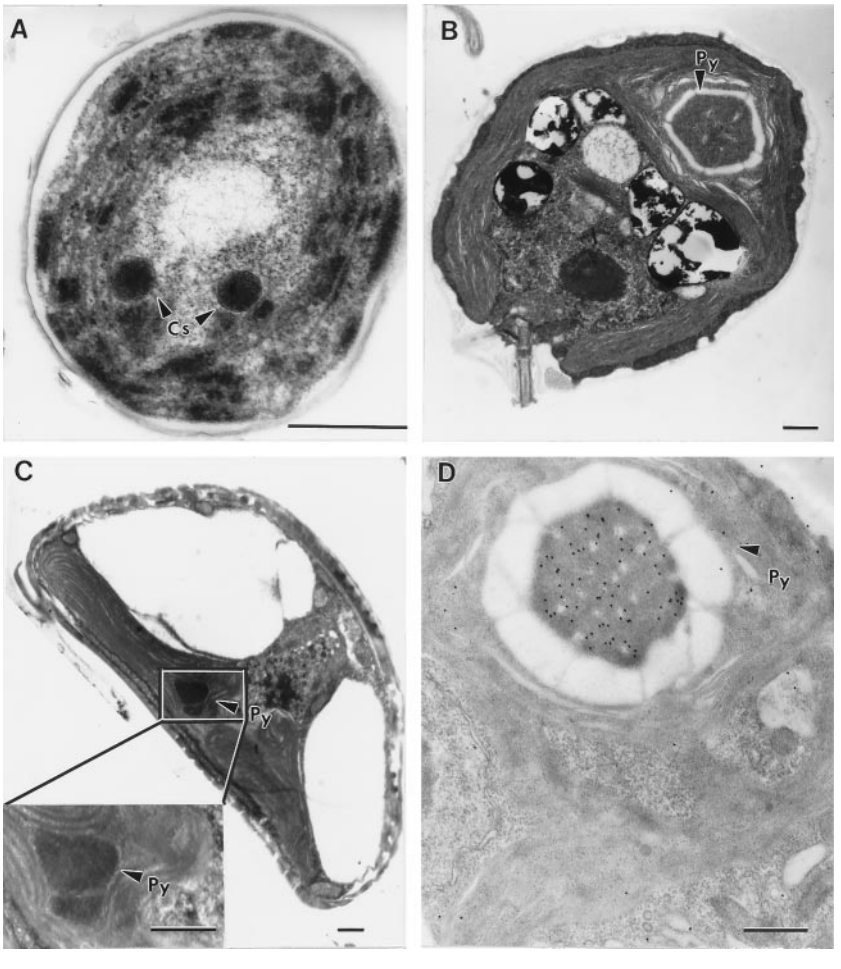

Figure 1. Carboxysomes and pyrenoids in different photosynthetic organisms. A, Electron micrograph of the cyanobacteria *Anabaena*; B, electron micrograph of the green alga *C. reinhardtii*; C, electron micrograph of the diatom *Amphora*; D, Immunogold labeling of the pyrenoid of *C. reinhardtii* with an anti-Rubisco antibody. Bars = 0.5 μm. Cs, Carboxysome; Py, pyrenoid.

Table I. *Location of Rubisco in organisms with different types of photosynthesis*

Photosynthesis Type	Ability to Concentrate CO_2?	Rubisco Location
C_3 photosynthesis (higher plants)	No	Chloroplast stroma of most cells in leaf
C_4 photosynthesis (higher plants)	Yes	Chloroplast stroma of bundle-sheath cells
Eukaryotic microalgae	Yes	Pyrenoid of the chloroplast
Cyanobacteria	Yes	Carboxysomes

research groups. Borkhsenious et al. (1998) found that the amount of Rubisco in the stroma varied with growth conditions: about 50% of the Rubisco was localized to the pyrenoid in *C. reinhardtii* cells grown on elevated CO_2 (5%, v/v). In contrast, they reported that when *C. reinhardtii* cells were grown under low CO_2 (ambient levels of CO_2 are considered low) more than 90% of the Rubisco was located in the pyrenoid. These results are consistent with those of Morita et al. (1997), who reported that 99% of the Rubisco was located in the pyrenoid in cells grown with ambient levels of CO_2.

C. reinhardtii concentrates CO_2 only when it is grown under low-CO_2 conditions. Because more than 90% of the Rubisco is localized to the pyrenoid under low-CO_2 conditions, one question is whether pyrenoidal Rubisco is active in CO_2 fixation or whether the pyrenoid is a storage body. In vitro measurements of Rubisco activity imply that the enzyme in the pyrenoid must be active to account for the levels of CO_2 fixation observed in *C. reinhardtii*. A specific localization of Rubisco to the pyrenoid is also compatible with the view that organisms that have CO_2-concentrating mechanisms specifically package Rubisco. In lichens and bryophytes there is a good correlation between the operation of a CO_2-concentrating mechanism and the presence of a pyrenoid (Smith and Griffiths, 1996). In cyanobacteria it appears that the CO_2 level is elevated within the carboxysome (Price et al., 1998), thus favoring carboxylation activity over the oxygenation activity of Rubisco. The pyrenoid may serve a similar function in *C. reinhardtii* and other microalgae.

THE ACCUMULATION OF HCO_3^-

The physiological evidence for the existence of CO_2 concentration in microalgae is 2-fold. First, algae are very efficient at pulling C_i out of the environment. They are much more efficient than would be expected, with cells showing an apparent affinity for CO_2 of about 1 μM versus the $K_m(CO_2)$ of Rubisco of about 20 μM. In some cases the growth conditions of the alga influences the cell's affinity for CO_2. Some species of algae, when grown on elevated CO_2 concentrations (10 times higher than ambient), are not efficient in their acquisition of C_i (Matsuda et al., 1998). However, if these same algae are grown on limiting CO_2 they become very efficient in CO_2 uptake and fixation. This implies that there are inducible transport mechanisms, because the amount of Rubisco does not change during adaptation from high- to low-CO_2 conditions.

Second, the accumulation of C_i within the cell can be measured directly. In the light, cyanobacteria can concentrate HCO_3^- within the cell more than 100-fold (Miller et al., 1990). Eukaryotic algae are not as efficient but can accumulate HCO_3^- at least 20-fold over ambient CO_2 levels. C_i transporters and CAs may enable the cells to accumulate HCO_3^- within the cell. The exact identity of the C_i transporters is still unknown, but recent work has identified some transporters that may play a significant role in the accumulation of C_i (Okamura et al., 1997).

In cyanobacteria difficulty in obtaining CO_2- and HCO_3^--transport mutants has been proposed to indicate the presence of multiple transporters for CO_2 and HCO_3^-. There is physiological evidence for three types of transporters: (a) a Na^+-independent HCO_3^- transporter, (b) a Na^+-dependent HCO_3^- transporter, and (c) a CO_2 transporter. Na^+-independent HCO_3^- transport under extreme C_i limitation (Espie and Kandasamy, 1992) and a difference in the magnitude of the requirement of Na^+ for HCO_3^- transport versus CO_2 transport (Miller et al., 1990) have been detected in *Synechococcus* PCC 7942. These data indicate the presence of either a Na^+/HCO_3^- symporter (Espie and Kandasamy, 1994) or the regulation of pH through Na^+/H^+ antiport mechanisms.

A mutant of *Synechococcus* PCC 7942, M42, has been shown to have a reduced affinity for HCO_3^-. The mutation in M42 has been shown to be in the gene cluster *cmpABCD*, which codes for a Na^+-independent, high-affinity HCO_3^- transporter induced under low C_i (Okamura et al., 1997). This is the first reported primary transporter for HCO_3^-, and belongs to the subfamily of ABC transporters also known as traffic ATPases (Higgins, 1992). The presence of an ABC-type transporter indicates that at high pH, when HCO_3^- is taken up, ATP may be the energy source for C_i uptake. A high-CO_2-requiring mutant of *Synechococcus* PCC 7942 has recently been characterized; it has a lesion in the gene *dc14* (Ronen-Tarazi et al., 1998), which encodes a putative Na^+-dependent HCO_3^- transporter. This transporter may be responsible for the fast induction response to low CO_2 reported from *Synechococcus* PCC 7942 and *Synechocystis* PCC 7002 (Sültemeyer et al., 1997).

Much less is known about the transport of C_i in microalgae. Extracellular C_i has to pass through at least two membrane systems to reach the site of carboxylation, which makes transport more complex than in cyanobacteria. At least two types of C_i uptake can be observed in microalgae. There is evidence for both direct transport of HCO_3^- and CA-facilitated diffusion of CO_2 across the membrane. The two membranes that we will consider as possible sites of C_i transport are the plasma membrane and the chloroplast envelope.

At the plasma membrane there is evidence for both HCO_3^- uptake and CA-facilitated diffusion. In *Scenedesmus obliquus* there is very good evidence that HCO_3^- is taken up directly by the cell (Thielmann et al., 1990). These cells can photosynthesize even when the pH is greater than 10 and HCO_3^- and CO_3^{2-} are the major C_i species. *Chlorella saccharophila* also appears to take up HCO_3^-, although CO_2 is its preferred C_i source (Williams et al., 1995).

The other major process by which microalgae take up C_i is through the uptake of CO_2. Many microalgae produce large amounts of CA when grown on limiting CO_2 (Raven, 1997). CA is a zinc metalloprotein, often located in the periplasmic space of the cell, that catalyzes the interconversion of CO_2 and HCO_3^- according to the following formula:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^-$$

Genes encoding periplasmic CAs have been identified in both *Dunaliella salina* and *C. reinhardtii* (Fujiwara et al., 1990). CA1, the periplasmic CA, has been identified as one of the prominent low-CO_2-inducible proteins in *C. reinhardtii*. The ability of microalgal cells to use external HCO_3^- for photosynthesis has been correlated with the presence of periplasmic CA. The presence of external CA inhibitors decreased the use of external C_i for photosynthesis (Moroney et al., 1985). The periplasmic CA probably increases the efficiency with which the cells can take in external C_i. This includes both the supply of CO_2 for diffusion across the plasma membrane and the supply of HCO_3^- for the plasma membrane's HCO_3^--transport system.

The chloroplast envelope is another possible location of HCO_3^- accumulation (Beardall, 1981). Intact chloroplasts isolated from *C. reinhardtii* and *Dunaliella tertiolecta* retain the ability to accumulate HCO_3^- when grown on low CO_2, and have the ability to concentrate CO_2. At low CO_2, *C. reinhardtii* induces the synthesis of LIP-36, a transport protein that is localized to the chloroplast envelope (Chen et al., 1997). LIP-36 belongs to a family of transport proteins that often act as exchangers (e.g. ATP for ADP transporters). It is possible that LIP-36 plays a role in HCO_3^- accumulation by the chloroplast, because chloroplasts with LIP-36 accumulate HCO_3^- and those without LIP-36, isolated from high-CO_2-grown cells, do not. The fact that LIP-36 is encoded by two separate genes (Chen et al., 1997) has made it difficult to obtain mutants devoid of this protein.

THE GENERATION OF CO_2 AT THE LOCATION OF RUBISCO

The generation of CO_2 at the location of Rubisco is accomplished through the action of a CA located at or near Rubisco. In cyanobacteria a CA is localized to the carboxysome (Price et al., 1992). Carboxysomes purified from *Synechocystis* species have significant CA activity. In *Synechocystis* 6803, for which the complete genome has been sequenced, only one CA gene has been identified. The role of this CA is the dehydration of accumulated HCO_3^- to form a localized, elevated concentration of CO_2 in the carboxysome. Loss of the carboxysomal CA through mutation leads to a cell that cannot grow well on limiting levels of CO_2 (Fukuzawa et al., 1992). In addition, cells missing the carboxysomal CA actually accumulate HCO_3^- to higher levels than wild-type cells, presumably because the cell can no longer convert the HCO_3^- to CO_2 for photosynthesis. In these CA-deficient cells, the CO_2-concentrating mechanism is still operational, but the final conversion of HCO_3^- to CO_2 is too slow.

It is noteworthy that CA activity is not found in the cytoplasm of cyanobacteria. Price and Badger (1989) demonstrated that transforming *Synechococcus* species with a human CA actually "short-circuits" HCO_3^- accumulation, and this transformant requires high CO_2 for growth. The human CA was localized to the cytoplasm and converted the accumulated HCO_3^- to CO_2. The CO_2 thus formed then leaked from the cell and could not be used efficiently for photosynthesis. From these studies it appears that the location of the internal CA is as important as the packaging of Rubisco.

In eukaryotic algae CA is often found inside the cell and in the periplasmic space. It is now clear that *C. reinhardtii* has at least five genes that encode CAs. Two of these genes, *Cah1* and *Cah2*, encode CAs that are directed to the periplasmic space (Fujiwara et al., 1990). Two more genes encode mitochondrial CAs (Eriksson et al., 1996). Recently, a fifth gene, *Cah3*, was found to encode a chloroplast CA (Karlsson et al., 1998). This CA has a leader sequence that directs the protein into the lumen of the thylakoid membrane. Pharmacological and genetic evidence indicates that Cah3 is essential in generating an elevated CO_2 concentration for Rubisco. It appears to play a role similar to that of the carboxysomal CA of cyanobacteria. This thylakoid CA is sensitive to sulfonamides, pharmaceuticals often used to inhibit mammalian CAs. Treatment of *C. reinhardtii* with sulfonamides that can enter the cell results in repression of CO_2 fixation (Moroney et al., 1985). Sulfonamides also severely inhibit photosynthesis in many other algae at low CO_2 concentrations, indicating that this thylakoid CA may be found in many algae. Furthermore, mutant strains of Cah3 are unable to grow at low CO_2, although the ability of these strains to accumulate HCO_3^- is not impaired. The thylakoid CA is thought to increase the concentration of CO_2 in the chloroplast by dehydration of the high concentration of HCO_3^- the cell accumulates there.

Chloroplast CAs from higher plants are quite different from the Cah3 protein of *C. reinhardtii.* Cah3 does not share any sequence similarity with higher-plant chloroplast CAs. The higher-plant enzymes are of the β-type and are found in the chloroplast stroma (Badger and Price, 1994). In contrast, Cah3 is of the α-type and is found in the thylakoid lumen (Karlsson et al., 1998). At this point no stromal CA has been found in an algal species that actively concentrates CO_2. It appears that a stromal CA might short-circuit the active accumulation of HCO_3^-. If CA were present in the chloroplast stroma it might convert accumulated HCO_3^- back to CO_2, allowing it to leak out of the cell before being fixed by Rubisco.

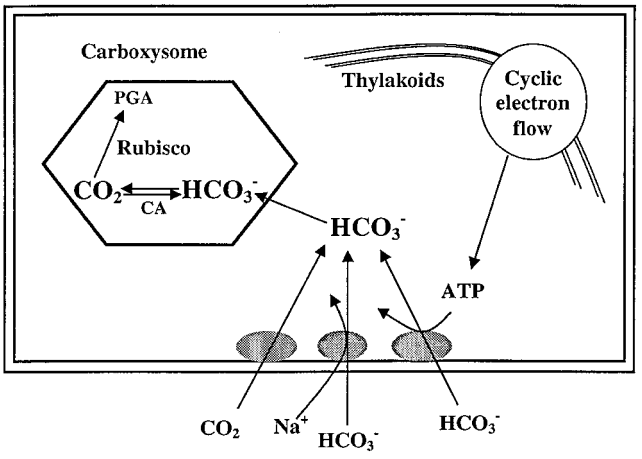

Figure 2. A model for CO_2 concentration in cyanobacteria. The font sizes of CO_2 and HCO_3^- indicate the relative concentrations of these C_i species. PGA, 3-Phosphoglyceric acid.

A MODEL FOR CO_2 CONCENTRATION

Even though the types of cells that possess CO_2-concentrating abilities are very different, they have certain properties in common that allow them to use CO_2 efficiently. The first property is the ability to accumulate HCO_3^- in some fashion. For most cyanobacteria and many eukaryotic algae, HCO_3^- can be transported into the cell directly. For other eukaryotic algae, particularly those that live in acidic environments, where the concentration of HCO_3^- is low, CO_2 is the C_i species that enters the cell and HCO_3^- is accumulated in the chloroplast. A second property is that Rubisco is usually packaged in a very specific way within the photosynthetic cell. Although it is possible that not every microalgal cell that concentrates CO_2 has a carboxysome or a pyrenoid, most cyanobacteria have carboxysomes and most microalgae have pyrenoids. The third property that appears to be common among these types of cells is the presence of a CA near the location of Rubisco. The CA supplies the Rubisco with CO_2 from the pool of HCO_3^-. Loss of this CA through mutation or inhibition greatly impairs the cell's ability to use external C_i for photosynthesis (Price et al., 1992; Karlsson et al., 1998).

A general model for CO_2 concentration in cyanobacteria is shown in Figure 2. Evidence for this model comes from physiological experiments and mutant analysis (Table II). In Figure 2 three different types of transporters are shown at the plasma membrane. It is very likely that there are a number of transporters important in HCO_3^- accumulation, because no single mutation has totally inhibited it. Recent work with the *Cmp* gene cluster of cyanobacteria has shown that high-affinity HCO_3^--transporter activity is lost if genes within this operon are deleted (Okamura et al., 1997). The Cmp operon appears to encode an ABC transporter with significant similarity to proteins known to transport small anions such as NO_3^- (Ogawa et al., 1998; Ohkawa et al., 1998). The fact that *Cmp* deletion mutants still retain the ability to grow on low HCO_3^- concentrations implies that other transporters remain to be identified. This is consistent with the multiple transport activities detected in the physiological experiments.

The amount of energy required for HCO_3^- uptake is not clear at present. Because ABC transporters require ATP, it is reasonable to assume that some ATP is used in HCO_3^- uptake (Fig. 2). Ogawa et al. (1998) have provided support for this contention by identifying a number of mutations that encode subunits of a NAD(P)H dehydrogenase. Deletions of these *ndh* genes lead to cells that require high CO_2 for photoautotrophic growth. The explanation for these mutants is that cyclic electron transport is disrupted in these cells such that too little ATP is made to support HCO_3^- transport. Mi et al. (1992) have also provided evidence that cyclic electron transport around PSI is required for HCO_3^- uptake.

Because Rubisco uses CO_2 and not HCO_3^-, the HCO_3^- accumulated by the cyanobacteria must be converted to CO_2 for fixation. As indicated in Table II, any disruption of the proper localization of Rubisco to the carboxysome in cyanobacteria leads to a cell that requires high CO_2 for photoautotrophic growth. One example of this is the loss of carboxysomes through loss of the carboxysomal shell proteins (Orús et al., 1995), in which case Rubisco is distributed in the cytoplasm. A similar situation occurs in the mutant EK6, which contains a 30-amino acid extension of the small subunit of Rubisco and has empty carboxysomes (Schwarz et al., 1995). Even though the kinetics of this

Table II. *High-CO_2-requiring strains and constructs of cyanobacteria*

Strain or Construct	Mutant Phenotype	Process Disrupted	Explanation
M3 and D4[a]	Lack of carboxysomes	Rubisco packaging	Carboxysomes fail to form; Rubisco is located in cytoplasm
Strain with *Rhodospirillum rubrum* Rubisco[b]	Rubisco in cytoplasm	Rubisco packaging	Bacterial Rubisco does not locate to carboxysome
Extension of Rubisco small subunit[c]	Empty carboxysomes	Rubisco packaging	Rubisco cannot package into carboxysome
Cmp deletions[d]	ABC transporter lost	HCO_3^- accumulation	High-affinity HCO_3^- transport lost
HCA II transformant[e]	CA in cytoplasm	HCO_3^- accumulation	Accumulated HCO_3^- leaks out as CO_2
IcfA deletion[f]	Loss of carboxysome CA	CO_2 generation	HCO_3^- not converted to CO_2 in carboxysome
Numerous *ndh* deletions[g]	Loss of NADH dehydrogenase	Energy mutations	Cyclic electron flow is disrupted

[a] Orús et al. (1995). [b] Pierce et al. (1989). [c] Schwarz et al. (1995). [d] Okamura et al. (1997); Price et al. (1998). [e] Price and Badger (1989). [f] Fukuzawa et al. (1992). [g] Ogawa et al. (1998); Price et al. (1998).

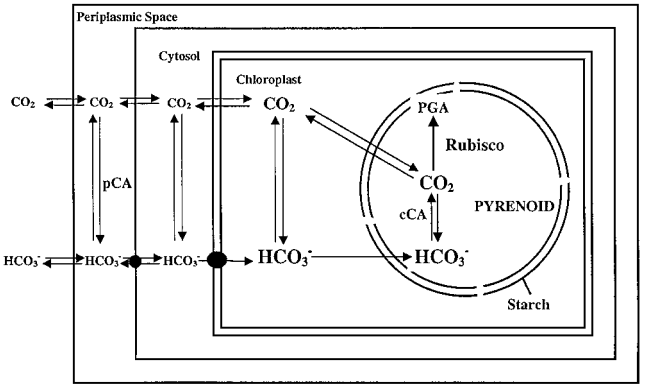

Figure 3. A model for CO_2 concentration in eukaryotic microalgae. The font sizes of CO_2 and HCO_3^- indicate the relative concentrations of these C_i species. cCA, Chloroplastic CA; pCA, periplasmic CA; PGA, 3-phosphoglyceric acid.

Rubisco appear normal, this strain requires high concentrations of CO_2 for normal growth. Again, the explanation appears to be that without an elevated CO_2 supply, the Rubisco is not packaged correctly into the carboxysome and ends up in the cytoplasm. Finally, the substitution of a bacterial Rubisco in place of the normal enzyme (Pierce et al., 1989) results in Rubisco free in the cytoplasm and in cells that require high CO_2 for growth.

The location of CA in cyanobacteria is also critical to the operation of the CO_2-concentrating mechanism. If the carboxysomal CA is inhibited or lost through mutation, the cell loses its ability to grow on low CO_2 concentrations. Therefore, the CA indicated in the carboxysome in Figure 2 is essential for the CO_2-concentrating mechanism, and its packaging is as important as the packaging of Rubisco.

A model of CO_2 concentration in eukaryotic algae is shown in Figure 3. This system is less understood because eukaryotic algae have more cellular compartments, are a very diverse group of organisms, and there are a limited number of systems in which molecular and genetic tools are available. However, the overall scheme of CO_2 concentration retains many similarities to the cyanobacterial model of active HCO_3^- accumulation, Rubisco packaging, and HCO_3^- dehydration in the chloroplast. In Figure 3 we have indicated both active uptake of HCO_3^- and diffusion of CO_2 across the plasma membrane, an uptake facilitated by the periplasmic CA. Microalgae also package their Rubisco in the pyrenoid, and deletion of the *rbcL* gene results in a strain without a pyrenoid (Table III).

The generation of CO_2 for Rubisco is also catalyzed by a specific CA, Cah3. Mutations in the gene encoding this chloroplastic CA require high CO_2 for photoautotrophic growth, and these mutants can be complemented by transforming the strain with the wild-type gene (Funke et al., 1997; Karlsson et al., 1998). In Figure 3 this CA is shown in the pyrenoid near Rubisco, but its exact location in relation to the pyrenoid has not been clearly established.

One important difference between algae, which concentrate CO_2, and C_3 plants, which do not, is the amount of CA activity in the stroma of the chloroplasts. In C_3 plants, there is a highly active, β-type CA in the chloroplast stroma (Badger and Price, 1994). In *C. reinhardtii* and other green algae there is very little, if any, stromal CA activity. In fact, the only chloroplast CA known is located in the thylakoid lumen (Karlsson et al., 1998). If the chloroplast is analogous to the cyanobacterial cytoplasm, a stromal CA might short-circuit the CO_2-concentrating mechanism. In cyanobacteria the insertion of human CA in the cytoplasm defeated the activity of the HCO_3^--accumulation system (Price and Badger, 1989). One prediction of the model shown in Figure 3 is that the presence of a CA in the chloroplast stroma might result in a cell that requires high CO_2 for growth.

AREAS OF CURRENT INTEREST

From the discussion above it is clear that there are still many unanswered questions about the mechanism by which microalgae accumulate C_i. The first challenge is to identify the other transport components of cyanobacteria and microalgae. In cyanobacteria a combination of better screening strategies for insertional mutants and the availability of the complete genome database for *Synechocystis* PCC 6803 should facilitate identification of the other C_i transporters and their mechanisms of operation. In microalgae the role of LIP-36 is being investigated. The recent development of several positive selectable markers for insertional mutagenesis in *C. reinhardtii* provides a powerful tool that will aid these studies. Insertional mutagenesis may be used not only for the identification of the C_i transporter, but also for the identification of other components involved in HCO_3^- accumulation, as well as the characterization of the roles played by these proteins.

A second important area of future interest is the role of the carboxysome and pyrenoid in CO_2 concentration. Carboxysomes are relatively well characterized in terms of their constituents, the genes that encode the proteins involved, and the effect of inactivation of these genes. However, the current evidence for the role of the pyrenoid in CO_2 concentration is circumstantial. The identification of mutants with disrupted or aberrant pyrenoids would help to clarify this issue.

A third area is the cost of CO_2 concentration. There is strong evidence for a light requirement in this process

Table III. *High-CO_2-requiring strains and Rubisco mutants of C. reinhardtii*

Strain or Construct	Mutant Phenotype	Process Disrupted	Explanation
Chloroplast *rbcL* mutations[a]	Lack of pyrenoid	Rubisco packaging	Loss of Rubisco; no pyrenoids formed
pmp mutant[b]	No HCO_3^- accumulation	HCO_3^- accumulation	Possible loss of transporter
ca-1 alleles[c]	Loss of chloroplastic CA	CO_2 generation	HCO_3^- not converted to CO_2 in chloroplast
cia-5[d]	Poor growth on low CO_2	Regulatory mutants	Does not adjust to low-CO_2 conditions

[a] Spreitzer et al. (1985); Rawat et al. (1996). [b] Spalding et al. (1983). [c] Funke et al. (1997); Karlsson et al. (1998). [d] Moroney et al. (1989).

(Raven, 1997). In cyanobacteria mutant analysis and antibody studies provide evidence for the energization of C_i accumulation through the NAD(P)H-dependent PSI cyclic electron flow (Mi et al., 1992). In microalgae the light requirement for CO_2 concentration may be attributable in part to the acidification of the lumen, because that is the location of Cah3 and, presumably, the site of the generation of CO_2 for Rubisco. As specific transport proteins are identified, the energy costs can be better estimated. It will be interesting to compare the cost of this process with C_3 and C_4 photosynthesis.

The regulation of the CO_2-concentrating mechanism is also an interesting challenge for future research. It is clear that the synthesis of many of the components of the CO_2-concentrating mechanism increases under low-CO_2 conditions (Beardall et al., 1998). Current evidence indicates that algal cells can "sense" the CO_2 level in the environment (Matsuda et al., 1998). The existence of mutants that fail to respond to low CO_2 (Table III) indicates that this signal transduction pathway can be identified through insertional mutagenesis studies. In addition, there are mutants of *Chlorella ellipsoidea* that express the CO_2-concentrating mechanism constitutively (Matsuda et al., 1998). A different approach has been taken by investigators who have linked the promoter regions for genes that respond to low-CO_2 conditions to reporter genes in *C. reinhardtii* (Eriksson et al., 1998). These chimeric genes respond to CO_2, and mutants have been found that fail to induce the reporter gene.

Another important current research topic is how organisms with a CO_2-concentrating mechanism will respond to increasing atmospheric CO_2 levels. For example, how will marine phytoplankton respond? If these organisms already possess an active CO_2-concentrating mechanism, then only a small increase in photosynthesis would be expected. On the other hand, if an algal species does not express the CO_2-concentrating mechanism under present atmospheric conditions, the increase in CO_2 might enhance its growth and photosynthesis. It is known that most algae, including coccoliths, diatoms, and cyanobacteria, have the ability to concentrate CO_2; however, little is known about whether these organism express the CO_2-concentrating mechanism in their native environments.

In conclusion, although much progress has been made in this field of study in the past few years, we are still a long way from complete characterization. The development of new tools and strategies will contribute to further progress in the elucidation of the C_i-accumulation mechanism in the microalgae.

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